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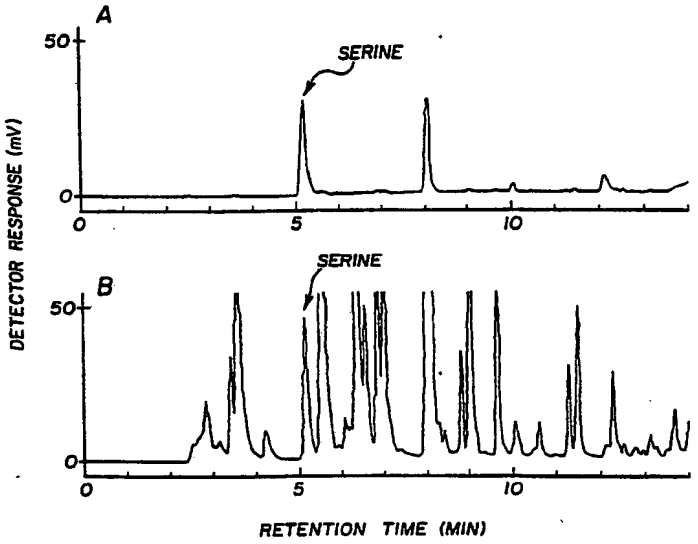

(57) Abstract

A variety of methods are provided for growing bacterial cells on lipids, acidic lipids, phospholipids, phosphatidylserine, or mucus, egg or milk fractions or subfractions. Pathogenic bacteria are preferred and include bacteria such as *Salmonella*, *Yersinia*, *Shigella*, *Campylobacter*, *Helicobacter*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*, *E. coli*, *Haemophilus*, *Mycobacterium*, *Proteus*, *Klebsiella*, *Neisseria*, *Branhamella*, *Bacteroides*, *Listeria*, *Enterococci*, *Vibrio*, *Yersinia*, *Bordetella*, *Clostridium*, *Treponema*, and *Mycoplasma*. The present invention also discloses methods for the selection of mutant strains which cannot grow in animals and use of such mutants as host cells for expression of cloned DNA molecules. In addition, methods are provided for the isolation of proteins whose expression is induced or enhanced by growth in the presence of phosphatidylserine or compositions containing phosphatidylserine. Such proteins have a number of uses, including as components of vaccines and as diagnostic markers. The present invention also provides methods for preparing bacteria or fractions thereof for use within cellular or acellular vaccines.

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Description

NUTRIENT PHOSPHOLIPIDS FOR PATHOGENIC BACTERIA

Technical Field

The present invention relates generally to bacteria, including pathogenic bacteria, and their growth on lipids. This invention is more particularly related to methods for growing bacterial cells, the production of mutant strains which cannot grow in animals and their use as host cells for expression of cloned DNA molecules, bacterial cells expressing proteins induced or enhanced by growth on lipids, and the use of such cells, fractions thereof, or individual proteins in vaccines.

Background of the Invention

Mucosal surfaces in various locations throughout the body have been suggested to serve as barriers to bacterial infection as well as sites for bacterial colonization. For example, a wide variety and as many as 400 different species of bacteria colonize the large intestines of both humans and animals. The large and small intestinal walls consist of an epithelium containing brush border epithelial cells and goblet cells which secrete a relatively thick (up to 400 μm) viscous, mucus covering. The epithelial cells synthesize glycoproteins and glycolipids which are integrated into the brush border membranes, thereby forming the epithelial cell glycocalyx. The mucus layer contains mucin, a 2×10^6 dalton gel-forming glycoprotein, and a large number of smaller components. Presumably, shed epithelial cells are the source of many of the smaller components of mucus. The intestinal mucus layer itself is in a dynamic state, continuously being synthesized and secreted by goblet cells and degraded to a large extent by intestinal indigenous microflora. Degraded mucus components are shed

into the lumen of the intestine and eventually find their way into feces.

For bacteria which colonize the intestinal tract (both pathogenic bacteria and those that constitute the normal flora), the interactions which occur at the mucosal surface are of vital importance in determining whether or not colonization will occur. In general, it appears that successful colonization depends upon the ability of bacteria to form a close association with the mucosal surface, to replicate and become established utilizing the nutrients available at the mucosal surface, and, in the case of the large intestine, to compete with the indigenous flora. More specifically, the major steps proposed to be involved in bacterial colonization of mucosal surfaces in general have been summarized to include: (a) chemotactic attraction of motile bacteria to the surface of the mucus gel, (b) penetration of and trapping within the mucus gel (which may be passive or can be promoted actively by bacterial motility and chemotaxis), (c) adhesion to the mucus gel or to the underlying mucosa-associated layers of the indigenous microflora, (d) adhesion to epithelial cell surface receptors, and (e) multiplication of the mucosa-associated bacteria. (R. Freter in Adhesion and Microorganism Pathogenicity, Ciba Foundation Symposium 80, Pitman Medical Ltd., London, p. 47, 1981.)

Currently, very little is known about bacterial-host interactions which occur at mucosal surfaces (e.g., of the large intestine) and which influence initiation and maintenance of colonization. In particular, for example, little is known regarding the interactions by which bacteria initiate and maintain a mucosal association in the large intestine (e.g., the genetic and metabolic events which accompany the bacterial response to the mucosal environment and the components and conditions which define specific niches in the mucosal environment). Such bacterial-host interactions are important because

colonization is an essential step in the pathogenicity of many pathogens, including enteric bacteria. Thus, there is a need in the art for methods and compositions based upon an understanding of interactions between pathogenic bacteria and host cells in the initiation and maintenance of colonization. The present invention fulfills these needs and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention provides a variety of methods and molecules related to bacteria, including pathogenic bacteria such as enteric bacteria and enteric invasive bacteria. In one aspect of the present invention, methods are provided for growing bacteria through the use of a variety of compositions and substances. In one embodiment, the composition consists of lipids, acidic lipids or phospholipids, each of which includes phosphatidylserine. In another embodiment, the composition comprises mucus, egg, or milk substantially free of proteins normally associated with the mucus, egg or milk, respectively. In other embodiments, the composition comprises mucus, egg or milk lipids substantially free of proteins normally associated with the mucus, egg or milk, respectively, or consists of mucus, egg or milk lipids. In other embodiments, the composition comprises mucus, egg or milk acidic lipids substantially free of proteins normally associated with the mucus, egg or milk, respectively, or consists of mucus, egg or milk acidic lipids. In yet other embodiments, the composition comprises mucus, egg or milk phospholipids substantially free of proteins normally associated with the mucus, egg or milk, respectively, or consists of mucus, egg or milk phospholipids. In a preferred embodiment, the substance consists of phosphatidylserine, which may be derived from mucus, egg or milk.

In another aspect of the present invention, methods are provided for selecting for a mutant strain of a bacteria. The methods comprise: exposing bacterial cells to phosphatidylserine, or to a composition consisting of lipids, acidic lipids or phospholipids, the composition including phosphatidylserine, or to a composition comprising mucus, mucus lipids, mucus acidic lipids or mucus phospholipids, the composition substantially free of proteins normally associated with the mucus, or to a composition comprising egg, egg lipids, egg acidic lipids or egg phospholipids, the composition substantially free of proteins normally associated with the egg, or to a composition comprising milk, milk lipids, milk acidic lipids or milk phospholipids, the composition substantially free of proteins normally associated with the milk, or to a composition consisting of mucus lipids, mucus acidic lipids, mucus phospholipids or mucus phosphatidylserine, or to a composition consisting of egg lipids, egg acidic lipids, egg phospholipids or egg phosphatidylserine, or to a composition consisting of milk lipids, milk acidic lipids, milk phospholipids or milk phosphatidylserine; and selecting for a mutant strain of the bacterial cells.

In yet another aspect, isolated mutant strains of bacteria produced by the methods of the present invention may be used to express a cloned DNA molecule introduced into the bacteria.

In a related aspect of the present invention, a method is provided for isolating bacterial proteins whose expression are induced or enhanced by growth in the presence of phosphatidylserine or a composition including phosphatidylserine. The method comprises: (a) growing bacteria in the presence of phosphatidylserine or a composition including phosphatidylserine under conditions and for a time sufficient to promote growth; (b) separating the proteins of the bacteria; (c) growing control bacteria under conditions and for a time

sufficient to promote growth, the control bacteria growing in media in the absence of phosphatidylserine or the composition; (d) separating the proteins of the control bacteria; (e) comparing the proteins separated in steps (b) and (d); and (f) isolating a protein from the bacteria, the protein absent from the control bacteria or present in lower amount in the control bacteria. Such proteins may be used as diagnostic markers to detect bacteria, especially pathogenic bacteria. Such proteins may also be used in a vaccine comprising a bacterial protein described above in combination with a pharmaceutically acceptable carrier or diluent.

In another aspect, the present invention provides methods for preparing bacteria or fractions thereof for use within a vaccine. In one embodiment, the method comprises: (a) growing bacteria in the presence of phosphatidylserine or a composition including phosphatidylserine under conditions and for a time sufficient to promote growth; and (b) isolating the bacteria. In another embodiment, the method comprises: (a) growing bacteria in the presence of phosphatidylserine or a composition including phosphatidylserine under conditions and for a time sufficient to promote growth; and (b) isolating the outer membranes from the bacteria. In another embodiment, the method comprises: (a) growing bacteria in the presence of phosphatidylserine or a composition including phosphatidylserine under conditions and for a time sufficient to promote growth; and (b) isolating the periplasm from the bacteria.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 graphically depicts the results of high-performance liquid chromatography of hydrolytically released serine from phosphatidylserine. Standard

phosphatidylserine, 1 μg (Panel a), and the acidic lipid fraction of mouse cecal mucus, 20 μg (Panel b), were hydrolyzed and derivatized with phenylisothiocyanate, and injected on a Supelcosil LC-18 (250 mm x 4.6 mm) column. The column was eluted with a linear gradient of ammonium acetate-trimethylamine as described by Bidlingmeyer et al. (J. Chromatogr. 336:93-104, 1984) using a UV detector operated at 254 nm.

Figure 2 pictorially depicts a comparison of proteins from the outer membrane and periplasm of Salmonella typhimurium or E. coli F-18 grown in L-broth or mucus dialysate. Panel A - Outer Membrane and Panel B - Periplasm. 100,000 cpm were added to each lane. Abbreviations: Std, protein standards; L-Ec, L-broth grown E. coli F-18; L-St, L-broth grown S. typhimurium; D-Ec, mucus dialysate grown E. coli F-18; D-St, mucus dialysate grown S. typhimurium.

Figure 3 pictorially depicts a comparison of proteins from periplasm of Campylobacter jejuni grown in mucus or Brucella broth. Lane 1 - C. jejuni periplasm from rabbit mucus grown cells probed with rabbit antiserum against rabbit mucus grown cells. Lane 2 - C. jejuni periplasm from Brucella broth grown cells probed with rabbit antiserum against rabbit mucus grown cells.

Detailed Description of the Invention

Determination of the components and conditions that promote bacteria-host interactions which occur at the mucosal surface and which are essential to initiation and maintenance of colonization has applicability to a wide range of settings. For example, mutant strains incapable of colonization may be selected for and molecules specific for colonization may be isolated. Such mutant strains and colonization-specific molecules have a variety of uses including as host cells with improved safety profiles for expression of a cloned DNA molecule and as a vaccine, respectively. As noted above, the present invention is

directed toward methods for growing bacterial cells and methods for selecting for a mutant strain of a bacterium which is incapable of growing on mucus. Such a mutant is incapable of colonization and/or is avirulent. In addition, such a mutant bacterium may be used for expression of a cloned DNA molecule which has been introduced into the bacterium. Mutant strains such as these would also be safe to be released into the environment. The present invention is also directed toward bacteria containing proteins whose expression has been induced or enhanced, and the use of such bacteria, fractions thereof or proteins in vaccines and as diagnostic markers.

The disclosure of the present invention shows that bacteria utilize lipids in mucus for growth and that the lipid fraction, subfractions thereof, or specific lipids and derivatives thereof may be used to support the growth of such bacteria. Bacteria which utilize lipids in mucus for growth include pathogenic bacteria, i.e., bacteria which are capable of producing pathological change or disease. Examples of pathogenic bacteria include Pseudomonas, Streptococcus, Staphylococcus, E. coli, Haemophilus, Mycobacterium, Proteus, Klebsiella, Neisseria, Branhamella, Bacteroides, Listeria, Enterococci, Vibrio, Yersinia, Bordetella, Clostridium, Treponema, and Mycoplasma. Bacteria which reside in a large or small intestine or a stomach are enteric bacteria and may be invasive or non-invasive. Examples of enteric invasive bacteria include Salmonella (such as S. typhimurium and S. cholera-suis), Yersinia (such as Y. entercolytica), Shigella (such as S. dysenteriae), Campylobacter (such as C. jejuni), and Helicobacter (such as H. pylori). Within the present invention, pathogenic bacteria are preferred.

The present invention provides methods for growing bacterial cells through the use of a variety of compositions and substances. In one embodiment, the

composition comprises mucus substantially free of protein normally associated with the mucus. Types of mucus include intestinal, gastric, and respiratory mucus. A mucus sample may be obtained from a variety of sources, including mammals such as humans, rabbits or mice, or birds such as chickens. For example, mucus may be prepared from mouse intestines. Briefly, animals, whose intake for the preceding 24 hours is only sterile water containing antibiotic, are sacrificed and the small intestines removed. After physical separation of contaminating material (such as partially digested food and feces), the mucus layer covering the mucosal surface is isolated (e.g., by scraping with a rubber spatula). The mucosal scrapings are separated from particulate and cellular material (e.g., by centrifugation) to yield mucus in the supernate. Substantially all of the proteins residing in a mucus sample may be generally removed by extraction(s) using salt, detergent and/or organic solvents. Briefly, for example, removal may be accomplished by extraction with chloroform/methanol (2:1) to yield mucus substantially free of proteins normally associated with mucus. An example of a protein normally associated with mucus is mucin, a 2×10^6 dalton gel-forming glycoprotein. It will be evident to those of ordinary skill in the art that residual amounts of proteins normally associated with mucus may remain after treatment of the mucus. The composition of the mucus which remains following treatment of the mucus to remove proteins should include lipids. The presence of lipids may be verified by well-known analytical techniques, such as chromatography (e.g., thin layer or high-performance liquid chromatography).

In another embodiment, the composition comprises mucus lipids substantially free of proteins normally associated with the mucus. Briefly, total lipids may be separated from mucus by organic solvent extraction of mucus. For example, separation may be accomplished by

extraction of mucus (dialyzed against water) using chloroform/methanol/water (e.g., 4:8:3) or chloroform/methanol (e.g., 2:1). A lipid fraction typically includes acidic lipids, neutral lipids, triglycerides, fatty acids, and glycolipids.

In another embodiment, the composition comprises mucus acidic lipids substantially free of proteins normally associated with the mucus. Briefly, acidic lipids may be separated from mucus by chromatography, such as high-performance chromatography and ion exchange chromatography. For example, separation may be accomplished by high-performance chromatography on silica Iatrobeads and ion exchange chromatography on DEAE cellulose. Acidic lipid fractions include phospholipids, sulfatides, and gangliosides.

In another embodiment, the composition comprises mucus phospholipids substantially free of proteins normally associated with the mucus. Briefly, phospholipids may be separated from mucus lipids by extraction or chromatography. For example, separation may be accomplished by ion exchange chromatography. A phospholipid fraction includes phosphatidylserine.

It may be desirable to add one or more substances to the compositions described above, e.g., for the purpose of enhancing growth. For example, one or more amino acids or proteins which are not normally associated with the mucus may be added to any of the compositions which are substantially free of proteins normally associated with the mucus. Similarly, other nutrients or salts may be added to any of the compositions.

In addition to the above-described compositions, substances suitable for use in methods for growing bacterial cells include individual phospholipids such as phosphatidylserine. Briefly, mucus phosphatidylserine may be separated from mucus phospholipids by chromatography. For example, separation may be accomplished by ion exchange chromatography. The disclosure of the present

invention shows that bacteria utilize phosphatidylserine in mucus for growth.

Alternatives to the isolation of lipid fractions or subfractions from mucus include other sources (such as avian egg or milk) for naturally derived lipid fractions or subfractions, the synthesis (e.g., chemically and/or enzymatically) of lipids for use within a composition as described above, or preparation of a composition using commercially available lipids (e.g., phospholipids are available from Avanti Polar Lipids, Inc., Alabaster, Alabama), or preparation of a composition using a combination of sources. Lipid fractions may be extracted from sources other than mucus, such as eggs, egg yolk extract (commercially available, e.g., from Miles Laboratories), or lyophilized milk, using the procedures described above for mucus. Acidic lipids and phospholipids may be separated from such lipid fractions using the procedures described above for mucus. It is desirable that the lipid fraction or subfraction, whether naturally derived or synthetically prepared, include phosphatidylserine. Briefly, phospholipids may be synthesized using glycol analogs (e.g., H. Eibl, "Synthesis of Glycerophospholipids," Chemistry and Physics of Lipids 26:405-429, 1980). For example, oleoxylpropandiol-(1,3) can be combined with phosphorous oxychloride and reacted with N-t-butyloxy-carbonyl-L-serine phthalimidomethylester in the presence of pyridine. The protecting groups are then removed by hydrazonolysis and treatment with formic acid. In addition, phosphatidylserine (e.g., derived from bovine brain) is commercially available. Alternatively, one or a combination of synthetic phosphatidylserines such as those with fatty acid chains containing C6, C8, C12 and C18 may be used.

Within the methods of the present invention for growing (e.g., culturing) bacterial cells, bacterial cells are exposed to one or more of the compositions or

substances described above. It will be evident to those of ordinary skill in the art that there are a variety of ways of exposing or contacting cells with a composition or a substance. For example, bacterial cells may be incubated with a composition or a substance. Typically, lipid fractions or purified lipid are dried under nitrogen, dispersed by sonication into HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-Hanks buffer (pH 7.4) at 1 mg/ml, and inoculated with about 2×10^4 colony forming units ("CFU") of bacteria per ml. Incubation takes place under conditions and for a time sufficient to permit growth of the bacterial cells. For example, cells may be incubated with a composition or a substance for 6 hours at 37°C. Growth of the bacterial cells may be determined and monitored qualitatively (e.g., by visualization or microscopic examination) or quantified (e.g., by plate counts).

In addition, the above-described compositions or substances may be prepared in the form of plates or broth to be used as bacteriological media within the methods of the present invention for growing bacterial cells. For example, for plates, 1.5% (w/v) of agar NOBL (Difco Laboratories, Detroit, MI) in HEPES-Hanks buffer (pH 7.4), containing 5 mg/ml of the detergent Brij 58 (Sigma Chemical Co., St. Louis, MO), is melted and allowed to cool to about 55°C-60°C. A substance such as phosphatidylserine (10-20 mg/ml) or a composition such as mucus lipids (5-10 mg/ml) is added and the plates are gently rotated until the agar solidifies. The plates may be stored at 4°C until used. Alternatively, 1% glucose can be substituted for the carbon source and 1 mg/ml of lipid added to supply the nitrogen source. For broths, for example, sterile lipid (5-25 mg/ml) is dispersed in HEPES-Hanks buffer (pH 7.4), containing 1-5 mg/ml of Brij 58. Such media (whether in plate, broth or other forms) is useful for culture, propagation and for culturing bacteria for antimicrobial testing.

In another aspect of the present invention, methods are provided for selecting for a "mutant strain" of a bacterium. As used herein, the term "mutant strain" refers to a bacterial strain which is unable to grow in mucus and incapable of intestinal or host colonization, whereas other strains are capable of growth in mucus. The methods comprise exposing bacterial cells to one or more of the compositions or substances described above and selecting for a mutant strain. Mutants of interest are unable to grow in the presence of phosphatidylserine as the sole source of carbon and nitrogen but continue to be able to grow utilizing D-glucose as the sole carbon source and ammonium chloride as the sole source of nitrogen. Briefly, for example, E. coli strains are mutagenized with pUJ10, a suicide plasmid, which contains a β-lactamase gene external to a TnphoA mobile element and which carries the transposase gene in cis but also external to the TnphoA mobile element (see, for example, de Lorenzo et al., J. Bacteriol. 172:6568, 1990). The TnphoA mobile element also contains a neomycin phosphotransferase gene, conferring kanamycin resistance. Since the transposase is physically separated from the transposable element of this vector, it is lost as the vector is lost, thereby resulting in stable transposition and in the isolation of stable mutants resistant to kanamycin but sensitive to ampicillin. Mutants are first screened on agar plates for the ability to grow utilizing glucose and ammonium chloride as the sole source of carbon and nitrogen. Those that are able to do so are tested, using 96 well polystyrene plates, for the ability to grow using phosphatidylserine as the sole source of carbon and nitrogen. Mutants that are unable to utilize phosphatidylserine for growth are tested for the ability to grow in mouse cecal mucus in vitro and to colonize the intestines of mice in vivo. Those that fail to grow in mouse cecal mucus and fail to colonize may be tested for the ability to grow in human colonic mucus in vitro.

Mutants that fail to grow in human mucus are preferred strains.

The mutant strains of the present invention have a variety of uses. For example, mutant strains may be used as host cells for expression of a cloned DNA molecule which has been introduced into the mutant bacterium. For this use, a mutant strain must be capable of expressing the cloned DNA molecule introduced into it. Expression capability may be confirmed by, for example, transforming random sequences of DNA isolated from the wild-type strains into a sample of cells of a mutant strain. Mutants containing sequences expressing the lipid transport proteins can be identified as now being able to grow utilizing lipids or phosphatidylserine as the sole source of carbon and nitrogen. To confirm that the recombinant plasmids contain DNA sequences encoding the lipid transport proteins, they may be extracted, transformed into a fresh transport mutant background, and shown to gain the ability to utilize the lipids and phosphatidylserine for growth. In addition, once a sequence is identified it can be subcloned and placed in the appropriate expression vector for isolation of large quantities of the protein.

The techniques for introducing DNA molecules into bacteria and expressing the proteins encoded thereby are well known to those of ordinary skill in the art (e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2 ed., Cold Spring Harbor Laboratory Press, Chapters 4 & 17, 1989). An expression vector may be constructed and then used to transform a microorganism for the expression and production of a protein. For example, recombinant plasmids capable of integration into a host mutant cell comprise a promoter followed downstream by a DNA sequence encoding a protein. It may be desirable to include a polyadenylation signal downstream from the DNA sequence. One embodiment of a method for producing a protein comprises introducing into a host mutant cell a

DNA sequence encoding a protein. The host cells are grown in an appropriate medium and the protein product encoded by the DNA sequence produced by the host cell is isolated. Examples of techniques known in the art include those disclosed in U.S. Patent Nos.: 4,440,859, issued April 3, 1984 to Rutter et al.; 4,530,901, issued July 23, 1985 to Weissman; 4,582,800, issued April 15, 1986 to Crowl; 4,677,063, issued June 30, 1987 to Mark et al.; 4,678,751, issued July 7, 1987 to Goeddel; 4,704,362, issued November 3, 1987 to Itakura et al.; 4,710,463, issued December 1, 1987 to Murray; 4,757,006, issued July 12, 1988 to Toole, Jr., et al.; 4,766,075, issued August 23, 1988 to Goeddel et al.; and 4,810, 648, issued March 7, 1989 to Stalker. It will be evident to those skilled in the art that it is not necessary to use the entire sequence when producing recombinant proteins. Further, DNA encoding one protein may be joined to a wide variety of other DNA sequences for introduction into a host cell.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognized by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Not all of the hosts may be transformed by the vector. Therefore, it may be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Mutant host cells of the present invention express protein(s) encoded by a cloned DNA molecule(s) which has been introduced into the mutant bacterium, but are unable to grow on mucus. Such cells are cultured by known techniques, and the proteins are recovered by known techniques. Depending upon the expression system used, the recombinant proteins expressed may be part of a fusion protein produced by the transformed host cells. Such proteins are recovered by known techniques, and the undesired part may be removed by known techniques. Alternatively, the fusion protein itself may be more immunogenic than the recombinant protein or polypeptide alone and, therefore, may itself be useful, e.g., in a vaccine.

Such mutant strains would be desirable hosts for recombinant DNA research as they grow well in laboratory media, but are unable to grow in mucus and colonize. Thus, these mutants would provide new cloning vectors with improved safety profiles for introduction into the environment. For example, a Salmonella or Pseudomonas mutant of the present invention is environmentally safe for release if it is non-invasive because of its inability to grow on host mucus and phosphatidylserine.

In a related aspect, the present invention provides methods for identifying or isolating bacterial proteins whose expression is induced, or at least enhanced, by growth under conditions which are associated with colonization. Certain proteins are either not expressed or only expressed in low levels under standard laboratory culture conditions. One method for identifying and isolating such proteins in substantially pure form is to first compare (e.g., by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) the proteins produced by a bacterium which is capable of utilizing phosphatidylserine for growth and which is grown in the presence of phosphatidylserine (i.e., grown on phosphatidylserine or on a composition including

phosphatidylserine), with the proteins produced by the bacterium when grown in the absence of phosphatidylserine ("control"). Based upon the comparison, proteins may be identified which are present in the bacterium grown in the presence of phosphatidylserine but which are absent in the control bacterium, or at least present in the control bacterium in lower levels. For example, as shown in Figure 2, a protein with a molecular mass of 45-46 kdal is expressed in the outer membranes of mucus grown cells, but is expressed only minimally in L-broth grown cells. Other examples of bacterial proteins whose expression is induced or enhanced, by growth of bacteria in phosphatidylserine or compositions containing phosphatidylserine, include E. coli outer membrane proteins of molecular mass 30-35 kdal, 40-42 kdal, and 40-100 kdal; E. coli periplasmic proteins of molecular mass 10-21 kdal, 23-45 kdal, and 45-100 kdal; Salmonella outer membrane proteins of molecular mass 30-35 kdal, 47 kdal, and 66 kdal; Salmonella periplasmic proteins of molecular mass of 35 kdal, 40 kdal, and 47 kdal; and Campylobacter outer membrane proteins of molecular mass 5-10 kdal, 35 kdal, 43 kdal, 47 kdal, 62 kdal, 84 kdal, and 180 kdal. It will be appreciated by those of ordinary skill in the art that, based upon the teachings described herein, especially when taken in combination with the knowledge in the art, additional proteins which are induced or enhanced by growth of bacteria in phosphatidylserine or compositions including phosphatidylserine can be readily identified and/or isolated from organisms other than those described above for illustrative purposes.

Following identification, such proteins may then be isolated in substantially pure form. For example, the proteins of a bacteria or a protein fraction (such as the membrane proteins) may be isolated from bacteria (e.g., by extraction and/or centrifugation) and separated from one another (e.g., by polyacrylamide gel electrophoresis). In parallel, the same procedure is performed using control

bacteria. It will be evident to those of ordinary skill in the art that separated proteins may be detected using a variety of techniques, such as stains, antibodies and the like. Detection of proteins which are present in small quantities in even the non-control bacteria may be accomplished by additional amplification of the detection of the separated proteins, e.g., by growing the bacteria in the presence of radioactive amino acids which metabolicly label the bacteria's proteins. Regardless of the particular means for detecting the separated proteins, a comparison of the separated proteins from control and non-control bacteria is made (e.g., visually, spectrophotometrically, etc.) to determine which proteins are present only in the non-control bacteria, or at least present in greater amounts in the non-control bacteria relative to the control bacteria. Based upon the identification of proteins whose expression is induced or enhanced in non-control bacteria, individual proteins may be isolated by a variety of techniques well known to those of ordinary skill in the art. Such techniques include extraction and/or chromatography. A substantially pure protein may be analyzed by various analytical techniques, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The proteins identified or prepared by the methods of the present invention may be used in vaccines to prevent diseases associated with pathogenic bacterial infections. Proteins identified or isolated by the methods of the present invention may be prepared by other methods for use in vaccines. For example, a protein may be prepared synthetically (chemically and/or enzymatically) or by recombinant technology, using methodologies well known to those in the art. Interference with the colonization of host cells by pathogenic bacteria (such as enteric invasive bacteria) is an effective basis for the prevention of the diseases which they cause. Administration of such proteins as a

vaccine leads to an immune response in which antibodies which bind to the protein are produced. These antibodies inhibit colonization of host cells by the pathogenic bacteria.

Alternatively, whole bacterial cells or fractions thereof, from bacteria grown using the methods of the present invention, may be used in vaccines. Briefly, whole cell vaccines are prepared from about 1×10^{11} bacteria which are harvested by centrifugation from media containing, for example, mucus, lipids derived from mucus, or phosphatidylserine, and washed three times with PBS. Bacteria are either inactivated by hearing at 56°C for 30 min. or by treating cells with 0.025 M formaldehyde at room temperature for 24 h and at 4°C for 24 h. The vaccine is typically administered in three oral doses at 2 week intervals or injected intramuscularly three times over a three to five week interval.

Similarly, outer membrane and/or periplasmic fractions, from bacteria grown using the methods of the present invention, may be used in vaccines. In addition, outer membrane and/or periplasm may be further fractionated to yield the respective protein subfractions. Such collections of outer membrane proteins and/or periplasmic proteins may be used in vaccines. Briefly, bacteria are grown, for example, in media containing mucus dialysate, lipids derived from mucus or phosphatidylserine. Whole cells are washed three times in PBS and sonicated three times on ice with microtip setting at 2 for 30 s each. After sonication, the cellular debris is removed by centrifugation at 5000 x g for 20 min. Collection of the total membrane fraction is facilitated by centrifugation at 100,000 x g for 60 min. The inner membrane is digested with 2% Sarkosyl in 7 mM-EDTA, pH 7.6, at 37°C with gentle rocking for 30 min. The suspension is centrifuged again at 100,000 x g for 2 h and the supernatant containing the periplasmic proteins and the pellet containing Sarkosyl insoluble outer membrane

proteins are collected. The proteins may be analyzed by SDS-PAGE. For example, samples (10 μg) are run in 10% polyacrylamide gels under reducing conditions by the standard procedure of Laemmli. Gels are stained with Coomassic blue stain or silver stain (Bío-Rad) to visualize the protein bands. Alternatively, bacteria are grown in media containing ^{35}S-methionine and ^{35}S-cysteine, membrane proteins are prepared as above and 100,000 cpm are added to each lane prior to SDS-PAGE analysis. Outer membrane proteins may also be prepared by lithium chloride-lithium acetate extraction (e.g., as described by Johnson et al., Infection and Immunity 57:1809-1815, 1989). Bacteria are suspended in buffer containing 0.2 M lithium chloride and 0.1 M lithium acetate. The pH is adjusted to 6.0 with acetic acid. Membrane vesicles are generated by shaking the cell suspension at 250 rpm at 45°C for 2 h in flasks containing 3-mm glass beads. Whole cells and debris are removed by centrifugation at 10,000 and 25,000 x g for 0 min. Outer membranes are washed once and pelleted by centrifugation at 100,000 x g for 1 h in Tris buffer (10 mM Tris, 100 mM NaCl, pH 8.0). The final pellet is suspended in sample buffer and may be processed for analysis by SDS-PAGE as described above.

In addition to the bacteria, fraction thereof or isolated protein (which function as antigen), it may be desirable to include other components in the vaccine, such as a vehicle for antigen delivery and immunostimulatory substances designed to enhance the protein's immunogenicity. Examples of vehicles for antigen delivery include aluminum salts, water-in-oil emulsions, biodegradable oil vehicles, oil-in-water emulsions, biodegradable microcapsules, and liposomes. Examples of immunostimulatory substances include N-acetylmuramyl-L-alanine-D-isoglutamine (MDP), lipopoly-saccharides (LPS), and glucan. It will be evident to those of ordinary skill in the art that a protein may be prepared synthetically and that a portion of the protein (naturally-derived or

synthetic) may be used. When a peptide of a protein is used, it may be desirable to couple the peptide hapten to a carrier substance, such as keyhole limpet hemocyanin.

The proteins of the present invention may also be used as diagnostic markers to detect bacteria, such as pathogenic bacteria. For example, phosphatidylserine may be immobilized on a solid support (such as beads) and contacted with a sample containing bacteria (e.g., in a bodily fluid such as urine). Phosphatidylserine may be immobilized onto a solid support (such as microtiter wells or chromatographic resins) by adsorption or covalent attachment. It will be evident that phosphatidylserine may be covalently attached in a variety of ways, including linker groups such as those available from Pierce Chemical Co. (Rockford, Ill.). A protein, whose expression in induced by phosphatidylserine, is then detected directly or indirectly. In conjunction with, or alternative to, contacting with phosphatidylserine, antibodies which specifically bind to such a protein may be utilized.

Polyclonal or monoclonal antibodies (MAbs) which are capable of specifically binding (i.e., with a binding affinity of about 10^6 liters per mole) a protein of the present invention may be produced. Briefly, polyclonal antibodies may be produced by immunization of an animal with a protein and subsequent collection of its sera. Immunization is accomplished, for example, by systemic administration, such as by subcutaneous, intraplenic or intramuscular injection, into a rabbit, rat or mouse. It is generally preferred to follow the initial immunization with one or more booster immunizations prior to sera collection. Such methodology is well known and described in a number of references.

MAbs may be generally produced by the method of Kohler and Milstein (Nature 256:495-497, 1975; Eur. J. Immunol. 6:511-519, 1976). Briefly, cells of lymph nodes and/or spleens of an animal immunized with a protein are fused with myeloma cells to form hybrid cell lines

("hybridomas" or "clones"). Each hybridoma secretes a single type of immunoglobulin specific for the protein, and, like the myeloma cells, has the potential for indefinite cell division. Suitable MAbs include those of murine or human origin, or chimeric antibodies such as those which combine portions of both human and murine antibodies (*i.e.*, antigen binding region of murine antibody plus constant regions of human antibody). Human and chimeric antibodies may be produced using methods well known by those skilled in the art. An alternative to the production of MAbs via hybridomas is the creation of MAb expression libraries using bacteriophage and bacteria (*e.g.*, Sastry et al., Proc. Natl. Acad. Sci. USA 86:5728-5732, 1989; Huse et al., Science 246:1275-1281, 1989).

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

PREPARATION OF FRACTIONS AND ISOLATED SUBSTANCES WHICH SUPPORT GROWTH OF BACTERIA

A. Mucus Isolation

Crude mucus was prepared from the intestines of 5- to 8-week-old CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Twenty-four hours before use the mice were deprived of food and given sterile water containing 0.5% (wt/wt) streptomycin sulfate. The following day the animals (usually four to six) were sacrificed, and the small intestines were removed and placed in sterile petri dishes containing HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-Hanks buffer (pH 7.4). The individual intestines were pooled and cut into 2- to 3-cm lengths. Any feces and partially digested food present were expressed from each section with a rubber spatula. The sections were

then transferred to a second set of petri dishes containing HEPES-Hanks buffer (pH 7.4) and split open with a scalpel. The split sections were agitated to remove any remaining debris and transferred to a third set of petri dishes. Each section was then gently scraped with a rubber spatula to remove the mucus layer covering the mucosal surface.

After the intestinal sections were discarded, the mucosal scrapings were centrifuged at 27,000 x g for 15 minutes to remove particular and cellular material. The resulting supernates contained the mucus.

B. Mucus Fractionation

1. Isolation of Total Lipids, Acidic Lipids and Neutral Lipids from Cecal Mucus

Dialysates of male CD-1 mouse cecal mucus, which support growth of E. coli and S. typhimurium as well as crude cecal mucus does *in vitro*, were used as the source of cecal mucus lipids. Cecal mucus (5 mg of protein) in 3 ml of HEPES-Hanks buffer, pH 7.4, was fractionated into one hundred 2.5 ml fractions on a Bio-Rad A5•m column (Wadolkowski et al., Infect. Immun. 56:1036-1043, 1988). The column was standardized before fractionation (Cohen et al., Infect. Immun. 48:139-145, 1985). Mucus fractions in which S. typhimurium SL5319 grew better than E. coli F-18 were pooled and dialyzed (Spectra/Por 3 dialysis tubing, 11.5 mm diameter, 3500 d cutoff, Los Angeles, CA) against 30 v of HEPES-Hanks buffer, pH 7.4, for 6 hours at 5°C. Dialysates were lyophilized and resuspended in their original volume. Lipids were extracted from mucus in chloroform/methanol/water (4:8:3) (Svennerhold et al., Biochim. Biophys. Acta 617:97-109, 1980) or in chloroform/methanol (2:1) (Slomiany et al., J. Biol. Chem. 253:3785-3791, 1978) and separated into neutral and acidic fractions by high-performance chromatography on silica Iatrobeads (Ando et al., Biochim. Biophys. Acta 424:98-105, 1976) and anion exchange chromatography on DEAE

cellulose, respectively (Rouser et al., p. 713-776, in G. Rouser, ed., Lipid Chromatographic Analysis, Vol. 3, Dekker, New York, 1976).

2. Determination of Amount of Phosphatidylserine in Cecal Mucus

To determine the amount of phosphatidylserine present in mouse cecal mucus, the acidic lipid fraction was hydrolyzed with 6N HCl for 18 hours at 120°C, and released serine was measured by the PICO-TAG method using phenylisothiocyanate (Cohen et al., Nature 320:769-770, 1986). Derivatized serine was separated and quantified by reverse phase high-performance liquid chromatography (HPLC) using a Supelcosil LC-18 column (Bidlinger et al., J. Chromatogr. 336:93-104, 1984). As shown in Figure 1, the HPLC profile obtained from the acidic lipid fraction (Panel b) included a peak with an identical retention value as the authentic phosphatidylserine standard (Panel a). Comparative analysis of the integrated peak areas indicated that 33.5 µg of phosphatidylserine was present per mg of total lipid extracted from mouse cecal mucus.

EXAMPLE 2

GROWTH OF BACTERIA IN MUCUS, FRACTIONS AND SUBSTANCES

Total lipids, acidic lipids, and neutral lipids of cecal mucus (prepared as described in Example 1) were dried under nitrogen and dispersed by sonication into HEPES-Hanks buffer, pH 7.4 at 1 mg per ml, and inoculated at about 2×10^4 cfu per ml. As shown in Table 1, S. typhimurium SL7312, a virulent strain, and E. coli F-18, a normal human fecal strain (Cohen et al., Infect. Immunon. 40:62-69, 1983), grew essentially as well utilizing cecal mucus total lipids as the sole source of carbon and nitrogen as in the cecal mucus dialysate itself, for the first six hours of incubation at 37°C.

Moreover, in the presence of total cecal mucus lipids, levels (cfu per ml) of about 20 percent of that reached in cecal mucus dialysates at 24 hours were observed (Table 1). The cecal mucus acidic lipids were also effective in promoting growth, whereas the neutral lipid fraction was relatively ineffective (Table 1). The pattern of growth of S. cholera-suis 735B, a pig pathogen, was identical to that of S. typhimurium in cecal mucus total lipids and acidic lipids. E. coli 933 EDL (O157:H7), a human enterohemorragic ("EHEC") strain, and E. coli A55 (O6, pap^+, hly^+), a pathogen causing human urinary tract infection ("UTI"), grew in the total and acidic lipid fractions essentially identically to E. coli F-18.

Phospholipids, sulfatides and gangliosides make up the majority of acidic lipids, whereas the majority of neutral lipids are neutral glycolipids. Therefore, several phospholipids (phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin; Avanti Polar Lipids, Inc., Alabaster, Alabama), purified mixtures of standard monosialylgangliosides (GM_1, GM_2, and GM_3; BioCarb Chemicals, Lund, Sweden), disialylgangliosides, (GD_{1a}, GD_{1b}, GD_2, and GD_3; BioCarb Chemicals), and a standard mixture of neutral glycolipids which contained galactosylceramide, lactosylceramide, globotriaosylceramide, globoside, and Forssman glycolipid (BioCarb Chemicals), were tested for their abilities to support growth of E. coli and Salmonella. Neither the E. coli nor Salmonella strains grew to any great extent on the mixtures of known gangliosides or neutral glycolipids (Table 2). Of the purified phospholipids used as the sole source of carbon and nitrogen, only phosphatidylserine supported growth of S. typhimurium SL7312 and E. coli F-18 to any great extent (Table 3). The other phospholipids were unable to support the growth of these bacteria. Similarly, phosphatidic acid, L-serine, a combination of phosphatidic acid and L-serine, and O-phospho-L-serine did not support growth,

although L-serine and O-phospho-L-serine did allow low levels of growth by 24 h (Table 3). Based upon the data, it appears that phosphatidylserine itself is transported and then metabolized. The EHEC and UTI strains also utilized phosphatidylserine for growth to the same extent as E. coli F-18, and S. cholera-suis 735B utilized phosphatidylserine to the same extent as S. typhimurium SL7312 (Table 4). Similarly, S. milwaukee, a human pathogen utilized phosphatidylserine for growth as efficiently as the other Salmonella strains. Phosphatidylserine, obtained, for example, from Avanti Polar Lipids (bovine brain, 99% pure) or from Sigma (St. Louis, MO, bovine brain, 98% pure), supported growth of the Salmonella and E. coli strains in a similar fashion. The present invention demonstrates that Salmonella and E. coli are capable of utilizing cecal mucus total lipids, cecal mucus acidic lipids, and phosphatidylserine, as the sole sources of carbon and nitrogen and that they do so without an extended lag period.

Table 1

Growth of S. typhimurium SL7312 and E. coli F-18 on cecal mucus lipids as the sole source of carbon and nitrogen

	CFU Relative to Input CFU[a]			
	6 h		24 h	
Substrate	F-18	SL7312	F-18	SL7312
Dialysate	264	1506	6436	12875
Total lipids[b]	620	916	1551	2125
Acidic Lipids	158	416	437	625
Neutral Lipids	0.14	1.83	0.02	4.44
HH[c]	0.55	0.85	2.53	6.55

[a] Input cfu per ml: E. coli F-18, 8.1×10^4; S. typhimurium SL7312, 6.6×10^4.

[b] Lipids were dispersed in HEPES-Hanks buffer, pH 7.4 at a concentration of 1 mg per ml.

[c] HEPES-Hanks buffer, pH 7.4.

Table 2

Growth of E. coli F-18 and S. typhimurium SL7312 on a mixture of standard acidic (gangliosides) and neutral glycosphingolipids

	CFU Relative to Input CFU[a]			
	6 h		24 h	
Substrate	F-18	SL7312	F-18	SL7312
Monosialylgangliosides[b]	0.69	0.89	0.10	3.25
Disialylgangliosides	0.75	1.52	0.02	5.45
Neutral Glycolipids	0.92	1.39	<0.02	5.16
HH[c]	0.85	0.81	0.21	1.69

[a] Input cfu per ml: E. coli F-18, 5.2×10^4; S. typhimurium SL7312, 6.4×10^4. Data are presented as the ratio of cfu at 6 h and 24 h to the input cfu.

[b] Mixtures were dispersed in HEPES-Hanks buffer, pH 7.4 at a concentration of 1 mg per ml.

[c] HEPES-Hanks buffer, pH 7.4.

Table 3

Growth of S. typhimurium SL7312 and E. coli F-18 on selected purified phospholipids

Substrate[b]	CFU Relative to Input CFU[a]			
	6 h		24 h	
	F-18	SL7312	F-18	SL7312
Phosphatidylserine	6.78	21.91	203	1319
Phosphatidylcholine	1.70	1.10	6.65	3.47
Phosphatidylethanolamine	0.74	0.49	0.52	0.70
Phosphatidylinositol	1.60	0.66	0.91	0.51
Sphingomyelin	0.65	0.36	0.30	0.12
Phosphatidic Acid + L-Serine	1.26	0.55	0.52	0.19
Choline Chloride	3.42	2.44	2.63	4.71
L-Serine	1.62	1.71	21.53	36.06
O-Phospho-L-Serine	2.41	3.62	31.13	36.52
HH[c]	1.39	0.78	0.74	0.45

[a] Input cfu per ml: E. coli F-18, 2.3×10^4; S. typhimurium SL7312, 4.7×10^4.

[b] Unless otherwise noted, all substrates were suspended in HEPES-Hanks buffer, pH 7.4 at 1 mg per ml.

[c] HEPES-Hanks buffer, pH 7.4.

Table 4

Growth of S. typhimurium SL7312 and S. cholera-suis 735B on selected purified phospholipids

	CFU Relative to Input CFU[a]			
	6 h		24 h	
Substrate[b]	F-18	SL7312	F-18	SL7312
Phosphatidylserine	29.18	28.10	222	533
Phosphatidylcholine	2.06	5.48	3.10	5.28
Phosphatidylethanolamine	0.43	0.81	1.42	1.95
Phosphatidylinositol	0.43	1.24	0.80	3.38
Phosphatidic Acid	0.20	1.38	1.04	2.33
HH[c]	0.37	1.48	0.88	2.48

[a] Input cfu per ml: S. typhimurium SL7312, 4.9×10^4; S. cholera-suis 735B, 2.1×10^4.

[b] Phospholipids were dispersed in HEPES-Hanks buffer, pH 7.4 at a concentration of 1 mg per ml.

[c] HEPES-Hanks buffer, pH 7.4.

From the foregoing, it will be evident that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

Claims

1. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of lipids including phosphatidylserine.

2. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of acidic lipids including phosphatidylserine.

3. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of phospholipids including phosphatidylserine.

4. A method for growing bacterial cells, comprising exposing bacterial cells to phosphatidylserine.

5. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising mucus substantially free of proteins normally associated with said mucus.

6. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising mucus lipids substantially free of proteins normally associated with said mucus.

7. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of mucus lipids.

8. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said

composition comprising mucus acidic lipids substantially free of proteins normally associated with said mucus.

9. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of mucus acidic lipids.

10. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising mucus phospholipids substantially free of proteins normally associated with said mucus.

11. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of mucus phospholipids.

12. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of mucus phosphatidylserine.

13. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising avian egg substantially free of proteins normally associated with said egg.

14. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising avian egg lipids substantially free of proteins normally associated with said egg.

15. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of avian egg lipids.

16. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said

composition comprising avian egg acidic lipids substantially free of proteins normally associated with said egg.

17. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of avian egg acidic lipids.

18. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising avian egg phospholipids substantially free of proteins normally associated with said egg.

19. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of avian egg phospholipids.

20. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of avian egg phosphatidylserine.

21. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising milk substantially free of proteins normally associated with said milk.

22. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising milk lipids substantially free of proteins normally associated with said milk.

23. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of milk lipids.

24. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said

composition comprising milk acidic lipids substantially free of proteins normally associated with said milk.

25. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of milk acidic lipids.

26. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition comprising milk phospholipids substantially free of proteins normally associated with said milk.

27. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of milk phospholipids.

28. A method for growing bacterial cells, comprising exposing bacterial cells to a composition, said composition consisting of milk phosphatidylserine.

29. The method of any of claims 1-28 wherein the bacterial cells are from bacteria selected from the group consisting of Salmonella, Yersinia, Shigella, Campylobacter, Helicobacter, Pseudomonas, Streptococcus, Staphylococcus, E. coli, Haemophilus, Mycobacterium, Proteus, Klebsiella, Neisseria, Branhamella, Bacteroides, Listeria, Enterococci, Vibrio, Yersinia, Bordetella, Clostridium, Treponema, and Mycoplasma.

30. A method for selecting for a mutant strain of a bacterium, comprising:

exposing bacterial cells to phosphatidylserine, or to a composition consisting of lipids, acidic lipids or phospholipids, said composition including phosphatidylserine, or to a composition comprising mucus, mucus lipids, mucus acidic lipids or mucus phospholipids, said composition substantially free of proteins normally associated with said

mucus, or to a composition comprising egg, egg lipids, egg acidic lipids or egg phospholipids, said composition substantially free of proteins normally associated with said egg, or to a composition comprising milk, milk lipids, milk acidic lipids or milk phospholipids, said composition substantially free of proteins normally associated with said milk, or to a composition consisting of mucus lipids, mucus acidic lipids, mucus phospholipids or mucus phosphatidylserine, or to a composition consisting of egg lipids, egg acidic lipids, egg phospholipids or egg phosphatidylserine, or to a composition consisting of milk lipids, milk acidic lipids, milk phospholipids or milk phosphatidylserine; and

selecting for a mutant strain of said bacterial cells.

31. The method of claim 30 wherein the bacterial cells are from bacteria selected from the group consisting of Salmonella, Yersinia, Shigella, Campylobacter, Helicobacter, Pseudomonas, Streptococcus, Staphylococcus, E. coli, Haemophilus, Mycobacterium, Proteus, Klebsiella, Neisseria, Branhamella, Bacteroides, Listeria, Enterococci, Vibrio, Yersinia, Bordetella, Clostridium, Treponema, and Mycoplasma.

32. An isolated bacterium mutant strain produced by the method of claim 30 wherein the bacterium is selected from the group consisting of Salmonella mutants, Yersinia mutants, Shigella mutants, Campylobacter mutants, Helicobacter mutants, Pseudomonas mutants, Streptococcus mutants, Staphylococcus mutants, Haemophilus mutants, Mycobacterium mutants, Proteus mutants, Klebsiella mutants, Neisseria mutants, Branhamella mutants, Bacteroides mutants, Listeria mutants, Enterococci mutants, Vibrio mutants, Yersinia mutants, Bordetella mutants, Clostridium mutants, Treponema mutants, and Mycoplasma mutants.

33. A mutant bacterium according to claim 32 for use within a method of expressing a cloned DNA molecule introduced into said bacterium.

34. A method for isolating a bacterial protein whose expression is induced or enhanced by growth in the presence of phosphatidylserine or a composition including phosphatidylserine, comprising:

(a) growing bacteria in the presence of phosphatidylserine or a composition including phosphatidylserine under conditions and for a time sufficient to promote growth;

(b) separating the proteins of said bacteria;

(c) growing control bacteria under conditions and for a time sufficient to promote growth, said control bacteria growing in media in the absence of said phosphatidylserine or said composition;

(d) separating the proteins of said control bacteria;

(e) comparing the proteins separated in steps (b) and (d); and

(f) isolating a protein from said bacteria, said protein absent from said control bacteria or present in lower amount in said control bacteria.

35. A protein identified by the method according to claim 34, said bacteria selected from the group consisting of Salmonella, Yersinia, Shigella, Campylobacter, Helicobacter, Pseudomonas, Streptococcus, Staphylococcus, E. coli, Haemophilus, Mycobacterium, Proteus, Klebsiella, Neisseria, Branhamella, Bacteroides, Listeria, Enterococci, Vibrio, Yersinia, Bordetella, Clostridium, Treponema, and Mycoplasma.

36. A vaccine comprising a protein identified by the method according to claim 34 in combination with a pharmaceutically acceptable carrier or diluent.

37. A method for preparing bacteria for use within a vaccine, comprising:

(a) growing bacteria in the presence of phosphatidylserine or a composition including phosphatidylserine under conditions and for a time sufficient to promote growth; and

(b) isolating said bacteria.

38. A vaccine comprising a bacterium prepared according to claim 37 in combination with a pharmaceutically acceptable carrier or diluent.

39. A method for preparing a bacterial outer membrane fraction for use within an acellular vaccine, comprising:

(a) growing bacteria in the presence of phosphatidylserine or a composition including phosphatidylserine under conditions and for a time sufficient to promote growth; and

(b) isolating the outer membranes from said bacteria.

40. The method of claim 39 further including, after the step of isolating, separating the proteins from said outer membranes.

41. An acellular vaccine comprising outer membranes prepared according to claim 39 or outer membrane proteins prepared according to claim 40, in combination with a pharmaceutically acceptable carrier or diluent.

42. A method for preparing a bacterial periplasmic fraction for use within an acellular vaccine, comprising:

(a) growing bacteria in the presence of phosphatidylserine or a composition including phosphatidylserine under conditions and for a time sufficient to promote growth; and

(b) isolating the periplasm from said bacteria.

43. The method of claim 42 further including, after the step of isolating, separating the proteins from said periplasm.

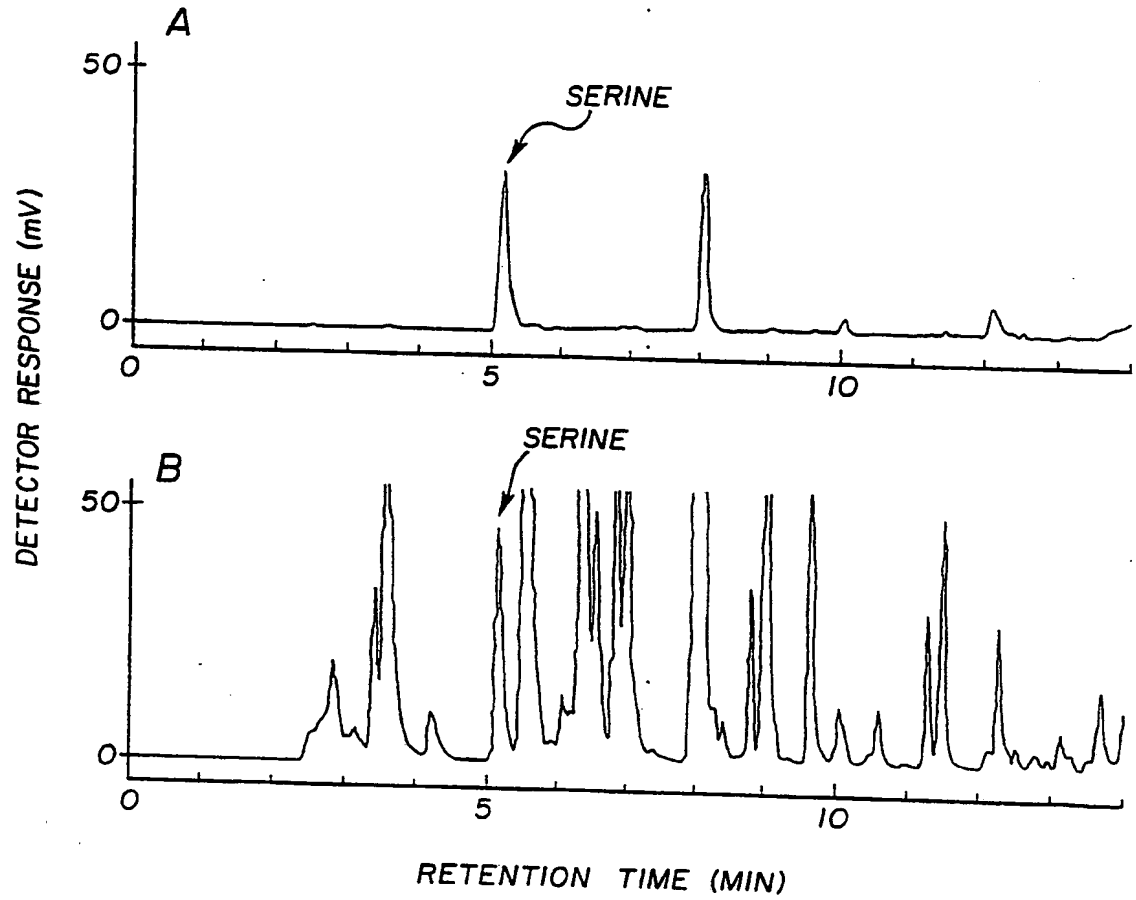
44. An acellular vaccine comprising periplasm prepared according to claim 42 or periplasmic proteins prepared according to claim 43, in combination with a pharmaceutically acceptable carrier or diluent.

45. An acellular vaccine comprising outer membranes prepared according to claim 39 and periplasm according to claim 42, in combination with a pharmaceutically acceptable carrier or diluent.

46. An acellular vaccine comprising outer membrane proteins according to claim 40 and periplasmic proteins according to claim 43, in combination with a pharmaceutically acceptable carrier or diluent.

47. The method of any one of claims 37, 39, 40, 42 or 43 wherein said bacteria are selected from the group consisting of Salmonella, Yersinia, Shigella, Campylobacter, Helicobacter, Pseudomonas, Streptococcus, Staphylococcus, E. coli, Haemophilus, Mycobacterium, Proteus, Klebsiella, Neisseria, Branhamella, Bacteroides, Listeria, Enterococci, Vibrio, Yersinia, Bordetella, Clostridium, Treponema, and Mycoplasma.

FIG. 1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DETECTOR RESPONSE (mV)
RETENTION TIME (MIN)

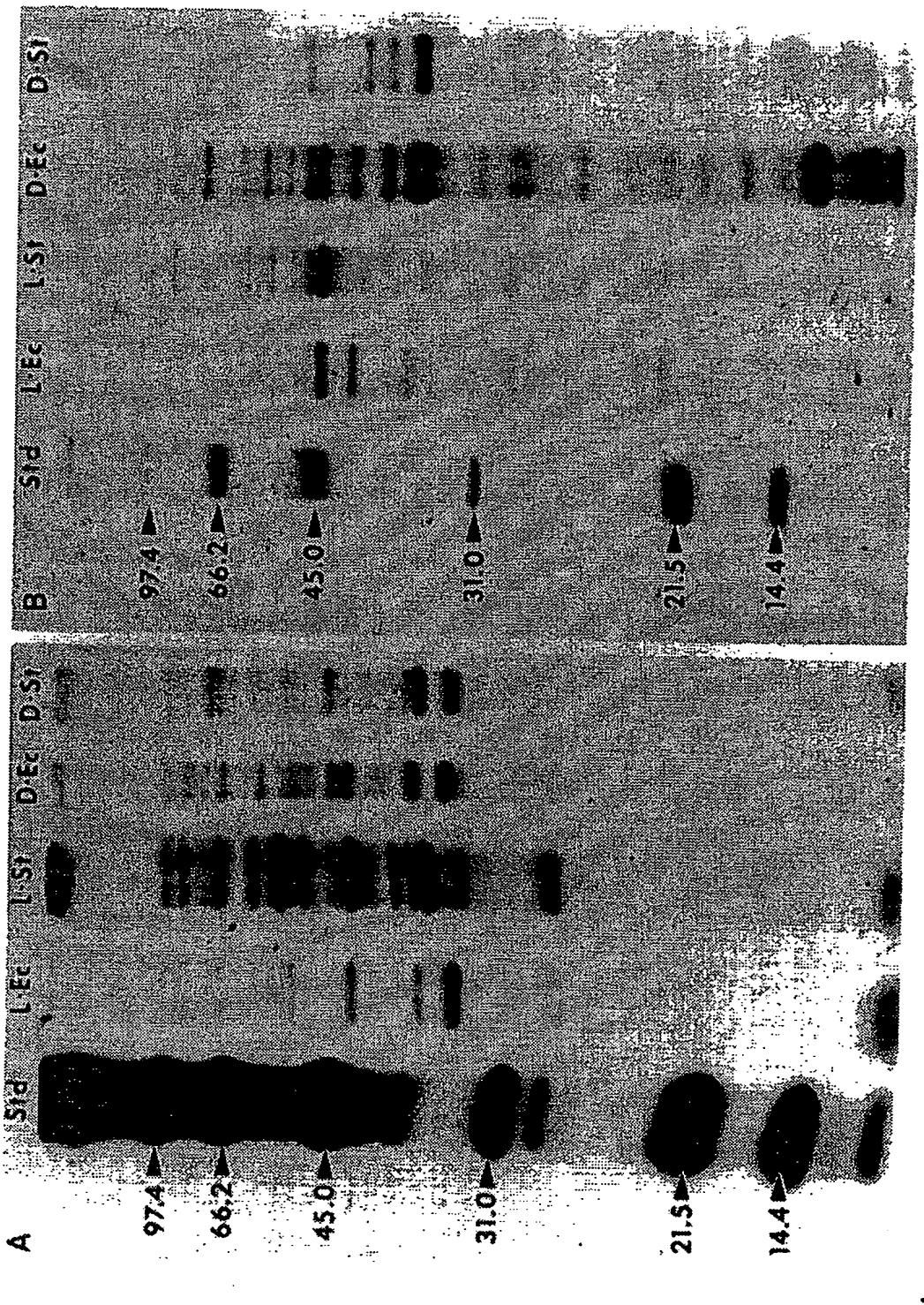
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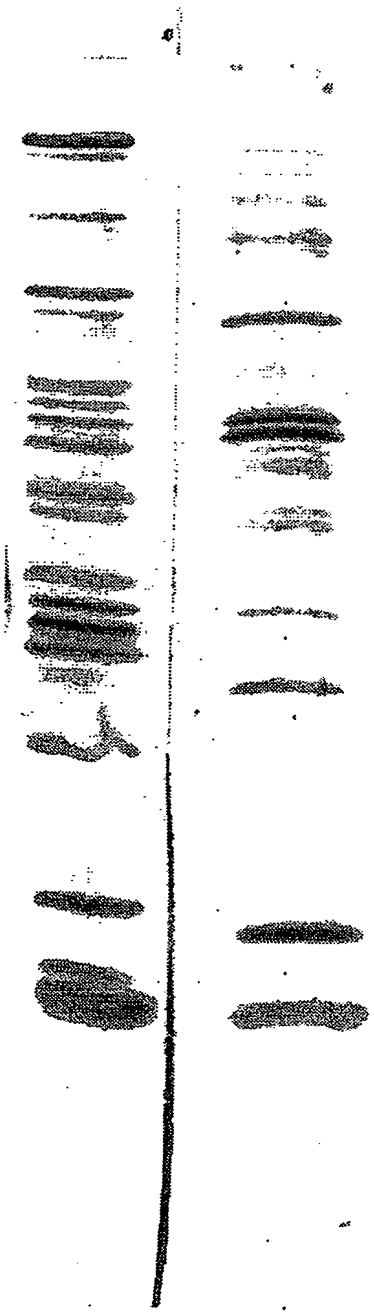
FIG. 2

FIG. 3



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/04053

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N1/20; C12N1/38; C12N15/01; A61K37/02
A61K39/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	INFECTION AND IMMUNITY vol. 40, no. 1, April 1983, AM. SOC. MICROBIOL.,BALTIMORE,US; pages 62 - 69 P.S.COHEN ET AL. 'Relationship between mouse colonizing ability of a human fecal Escherichia coli strain and its ability to bind a specific mouse colonic mucous gel protein' cited in the application see page 63, left column, paragraph 2 - page 65, left column, paragraph 3 --- -/--	1-47

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 JULY 1993	30. 07. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	HORNIG H.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	INFECT. IMMUN. vol. 56, no. 5, May 1988, AM.SOC.MICROBIOL.,BALTIMORE,US; pages 1030 - 1035 E.A. WADOLKOWSKI ET AL. 'Colonization of the streptomycin-treated mouse large intestine by a human Escherichia coli strain: Role of growth in mucus' cited in the application see page 1030, line 1 - line 16	1-47
A	INFECT. IMMUN. vol. 56, no. 9, September 1988, AM.SOC.MICROBIOL.,BALTIMORE,US; pages 2209 - 2217 B.A. MCCORMICK ET AL. 'Roles of motility, chemotaxis, and penetration through and growth in intestinal mucus in the ability of an avirulent strain of Salmonella typhimurium to colonize the large intestine of streptomycin-treated mice' cited in the application see page 2209, line 1 - line 19	1-47
P,X	INFECT. IMMUN. vol. 60, no. 9, September 1992, AM. SOC.MICROBIOL.,BALTIMORE, US; pages 3943 - 3946 H.C.KRIVAN ET AL. 'Phosphatidylserine found in intestinal mucus serves as a sole source of carbon and nitrogen for Salmonellae and Escherichia coli' see page 3943, left column, line 1 - page 3945, left column, line 15	1-12,29